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A New RNA Synthetic Method with a 2′-*O*-(2-Cyanoethoxymethyl) Protecting Group

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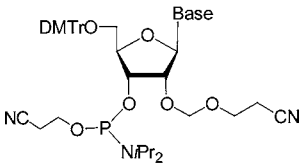
ABSTRACT



Base =U, A^{Ac}, C^{Ac}, G^{Pac}

A novel method for the synthesis of RNA oligomers with 2-cyanoethoxymethyl (CEM) as the 2′-hydroxyl protecting group has been developed. The new method allows the synthesis of oligoribonucleotides with an efficiency and final purity comparable to that obtained in DNA synthesis.

The recent discovery of RNA interference (RNAi) has opened new windows on the biology and potential therapeutic applications of short RNA molecules such as small interfering RNA (siRNA).[1,2] This in turn has led to increased interest in the chemical synthesis of RNA. From the outset of research on RNA synthesis, it has been appreciated that the single most demanding problem is the selection of an appropriate protecting group for the 2′-hydroxyl function. This protecting group must be stable throughout the solid-phase synthetic cycle, yet it must be readily removable under conditions under which the final RNA product is completely stable.[3] The conventional synthetic strategy is to use a 4,4′-dimethoxytrityl (DMTr) group or a silyl derivative[4] to protect the 5′-hydroxyl position and then to select a 2′-hydroxyl protecting group compatible with this, such as a fluoride-cleavable silyl ether,[5] a photolabile moiety,[6] or an acid-cleavable acetal.[7] In particular, *tert*-butyldimethylsilyl (TBDMS), a silyl ether group, is a popular protecting group whose amidite, which is used in solid-phase synthesis, has been commercially available for several years.

Although the TBDMS method gives RNA of modest purity in modest yield, it is not a robust method in the sense that both the purity and the yield are sensitive to small variations in the experimental conditions. Furthermore, the TBDMS group is associated with relatively long coupling times[8a] and insufficiently high coupling yields,[8b] though improvements

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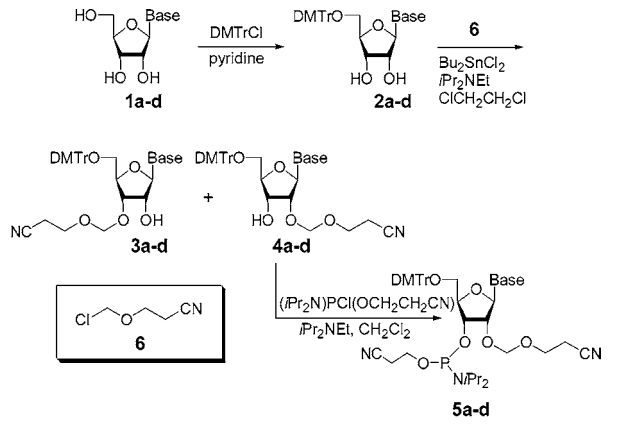
based on the use of more powerful activation reagents or sterically less demanding 2′-hydroxyl protecting groups have been proposed.[8b] Recently, to resolve these problems, two new protecting groups, bis(2-acetoxyethyloxy)methyl (ACE)[4] and triisopropylsilyloxymethyl (TOM),[9] were developed. However, though these protecting groups represent major improvements in the synthesis of RNA oligonucleotides,[10] they still leave a certain amount to be desired. Thus, the synthesis of ACE-amidites is relatively complex[3] (though they are now commercially available), and automated synthesizers require special modification for use with them because of the incompatibility of glass materials with triethylamine trihydrofluoride, the 5′-desilylation reagent. TOM-protected oligonucleotides, meanwhile, are not readily amenable to routine analysis and purification by HPLC because of the hydrophobic nature of the silyl group.

Pfleiderer and co-workers[11] investigated a wide variety of acid-cleavable acetal derivatives as protecting groups for the 2′-hydroxyl function. In particular, they identified benzyl acetals as promising protecting groups. They also observed that acetal derivatives with electron-withdrawing substituents, for example, 1-(2-cyanoethoxy)ethyl, were cleaved by fluoride anion under aprotic conditions in a side reaction during the synthesis of the 2′-*O*-protected nucleoside, though both these workers and Wada's group[12] have described a way to suppress this side reaction by the addition of AcOH. Furthermore, Gough et al.[8] have introduced the fluoride-cleavable 4-nitrobenzyloxymethyl protecting group, which allows analysis and purification of the protected oligonucleotides by HPLC.

In our search for a synthetic method that would exclude the side reaction, minimize steric hindrance, avoid the generation of asymmetric centers, and allow ready cleavage of the protecting group from the final product by fluoride anion, we decided to focus on the introduction of electron-withdrawing substituents into formaldehyde acetal type protecting groups. This approach led to the development of a novel protecting group, 2-cyanoethoxymethyl (CEM). In the present communication, we report on the use of CEM-amidite chemistry to synthesize homo- and mixed-base RNA oligomers up to 55 bases in length, and we show that the RNA product is obtained in high yield and high purity. Our syntheses proceeded about as readily and efficiently as DNA synthesis, demonstrating the potential usefulness of the CEM protecting group in solid-phase RNA synthesis.

First, we synthesized the phosphoramidite **5a**−**d** according to Scheme 1. Starting with a suitable base-protected nucleoside, **1a**−**d**,[9,13] we derivatized the 5′-hydroxyl group with DMTr and then the 2′-hydroxyl group with CEM. CEM

Scheme 1



1- 5: Base =U (a); A^{Ac}(b); C^{Ac}(c); G^{Pac}(d)

derivatization was carried out via the 2′,3′-*O*-dibutylstannylidene intermediate,[9] which was treated with the novel alkylating agent 2-cyanoethyl chloromethyl ether (**6**; CEM-Cl) to give a mixture of the 3′-*O*- and 2′-*O*-CEM derivatives (**3a**−**d** and **4a**−**d**). By using 1.0−1.3 equiv of **6**, the desired compound **4a**−**d** was obtained in 29−51% yield, and for all compounds except **4b** the 2′-isomer **4** was obtained in higher yield than the undesired 3′-isomer **3** (see Supporting Information). With G as the base, the ratio of the 2′-isomer (**4d**) to the 3′-isomer (**3d**) was the highest, at 3.0. After isolating the 2′-*O*-CEM derivative **4a**−**d** by silica gel column chromatography, we carried out phosphitylation of the 3′-hydroxyl group to obtain the corresponding amidite **5a**−**d**. The yields of the amidites have not yet been optimized, and this route is still under investigation to try to improve the regioselectivity of the alkylation reaction. CEM-Cl itself was prepared via the Pummerer reaction. Briefly, 3-hydroxypropionitrile and dimethyl sulfoxide were reacted to give the methylthiomethyl ether, which was then treated with sulfuryl chloride to give CEM-Cl. Both steps proceeded in reasonable yield (70−85%; see Supporting Information).

Turning to the synthesis of oligomers, we initially synthesized a uridine homo-oligomer, U_{40} (**7**), by our CEM method. Commercially available 2′- or 3′-*O*-benzoyl-rU controlled-pore glass (CPG) was used as the solid support and 5-ethylthiotetrazole as the activator. Solid-phase synthesis was carried out on an Applied Biosystems Expedite model 8909 DNA synthesizer on a 1-μmol scale with a coupling time of 150 s. Cleavage from the resin and deprotection of the phosphate moiety were carried out by treatment with concentrated ammonia in EtOH at 40 °C for 4 h. At this stage, the 2′-*O*-CEM-protected U_{40} can be monitored by HPLC (Figure 1A), because the 2′-*O*-CEM protecting group is relatively hydrophilic compared with the TBDMS group. (We initially tried $MeNH_2$ in EtOH/water for the cleavage/deprotection step, because this is the deprotecting reagent usually used. However, under these conditions we observed substantial loss of the CEM group

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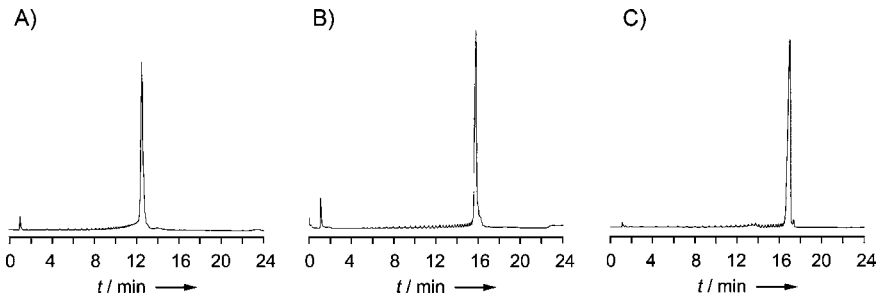

Figure 1. HPLC analysis of homo RNA and DNA. (A) Unpurified 2′-*O*-CEM-protected U_{40}, DNA Pac PA-100 anion-exchange column (4.6 mm × 250 mm; Dionex); buffer A, 10% CH_3CN, 25 mM Tris-HCl, pH 8.0; buffer B, 10% CH_3CN, 25 mM Tris-HCl, pH 8.0, containing 700 mM $NaClO_4$; gradient from 5% to 50% buffer B in 20 min; flow rate, 1.5 mL/min; 50 °C. (B) Unpurified fully deprotected U_{40} (**7**), gradient 5% to 50% buffer B in 20 min. (C) Unpurified fully deprotected dT_{40} (DNA). HPLC conditions: buffer A, 25 mM Tris-HCl, pH 8.0; buffer B, 25 mM Tris-HCl, pH 8.0, containing 700 mM $NaClO_4$; gradient from 10% to 30% buffer B in 20 min; flow rate, 1 mL/min; 40 °C. UV detection was at 260 nm.

accompanied by chain cleavage. When we used ammonia instead, the loss of CEM was less than 5% and no chain cleavage was observed.) In the next step, the 2′-*O*-CEM protecting group was completely removed by treatment with 1 M TBAF/THF for several hours. (With other deprotecting reagents that we tried, for example, $Et_3N{\cdot}3HF$, the CEM group was not removed.) HPLC profiles of U_{40} after deprotection are shown for the fully deprotected crude RNA prepared by the CEM method (Figure 1B) and for DNA of the same chain length (dT_{40}; Figure 1C). Comparison of the HPLC profiles of the entire crude reaction mixtures shows that the CEM method gave RNA of more than 80% purity, as obtained in DNA synthesis.

We next synthesized RNA oligonucleotides incorporating all four bases (Table 1). The phenoxyacetyl group was used as the base-protecting group for G and the acetyl group as the base-protecting group for A and C. For capping, phenoxyacetic anhydride was used instead of acetic anhydride to prevent replacement of the phenoxyacetyl group of protected G by the acetyl group, a side reaction reported by Chaix et al.[13b]

The RNA attached to CPG was initially treated similarly to the way we treated U_{40} (**7**) at this stage. However, during subsequent removal of the CEM group, a side reaction occurred that MALDI-TOF MS evidence suggested was the formation of cyanoethyl adducts (data not shown). To try to suppress the formation of these adducts, we tested various scavengers in various combinations and found that 10% *n*-propylamine and 1% bis(2-mercaptoethyl) ether in 1 M TBAF/THF gave the desired fully deprotected adduct-free product. A HPLC profile of **8** (55mer) in the entire crude reaction mixture from the CEM method is shown in Figure 2A. We emphasize that this HPLC profile shows absolutely unpurified RNA oligomer. As observed for the synthesis of the uridine homo-oligomer (**7**), the CEM method also yielded a high-purity, high-yield product in the synthesis of mixed-base RNA oligomers. Anion-exchange chromatography of the reaction mixture resulting from the CEM method with broad pooling of the main peak fractions followed by desalting by dialysis was sufficient to give highly purified RNA oligomer (Figure 2B). No modified bases were detected on HPLC analysis of enzymatically digested RNA oligomer **8** (Figure 2C). The identity of **8** was confirmed when its mass was measured by MALDI-TOF MS (positive-ion mode) at 17474.6 ($[M + H]^+$; calcd, 17476.6). As far as we know, a 55mer is the longest synthetic RNA oligomer whose structure has been confirmed by physicochemical data.

In conclusion, we have found that 2-cyanoethoxymethyl (CEM), a new 2′-hydroxyl protecting group that is readily removable under TBAF deprotection conditions, allowed RNA synthesis to be carried out much more simply and easily than by the conventional TBDMS methodology. RNA synthesis by the CEM method proceeded with coupling yields greater than 99% at each step while giving a high overall yield and a high purity, and the method is comparable in efficiency with DNA synthesis. We would like to

Table 1. Isolated Yields in the Synthesis of Oligonucleotides

entry	sequence	yield
1	5′-UUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUU-3′ (**7**; 40mer)	254 ODU (65%)
2	5′-UGAAUACAAAUCACAGAAUCGUCGUAUGCAGUGAAAACUCUCUUCAAUUCUUUAdT-3′ (**8**; 55mer)	83 ODU (15%)
3	5′-AAUCACAGAAUCGUCGUAUGCAGUGAAAACUCUCUUCAAdT-3′ (**9**; 40mer)	75 ODU (19%)
4	5′-ACAUCACUUACGCUGAGUACUUCGAAAUGU-3′ (**10**; 30mer)	92 ODU (31%)
5	5′-CUUACGCUGAGUACUUCGAU-3′ (**11**; 20mer)	112 ODU (58%)

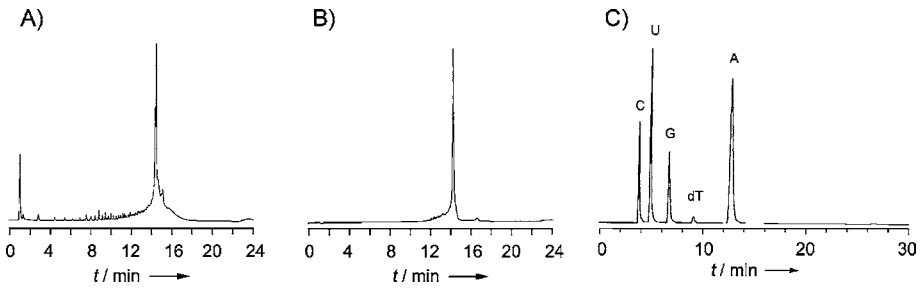

Figure 2. HPLC analysis of mixed-base RNA. (A) Unpurified fully deprotected RNA (**8**; 55mer). (B) Purified fully deprotected RNA (**8**; 55mer). HPLC conditions were the same as in Figure 1A except that the linear gradient was from 5% to 40% buffer B in 20 min. (C) HPLC analysis of RNA (**8**; 55mer) after enzymatic digestion: Develosil ODS-UG-5 reverse-phase column (4.6 mm × 250 mm); buffer, 5% MeOH, 5 mM $(n\text{Bu})_4\text{NHSO}_4$, 50 mM phosphate, pH 7.5; flow rate, 1 mL/min; 35 °C. UV detection was at 260 nm. As expected, a small dT peak derived from the 3′ end of **8** was observed.

emphasize that, unlike ACE chemistry, CEM chemistry is compatible with standard unmodified DNA synthesizer equipment. Furthermore, our results suggest that the CEM method, after it is suitably optimized, will have the potential for application to the synthesis of very long RNA oligonucleotides. An additional attractive feature of the method is that the CEM alkylating agent used can be synthesized by a simple two-step procedure from a moderately priced starting material, 3-hydroxypropionitrile, and so the method may be scaled up at reasonable cost.

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Supporting Information Available: Data on the other RNA oligomers in Table 1, as well as a detailed description of the experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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